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Role of peripheral polyamines in the development of inflammatory pain

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ABSTRACT

Polyamines (putrescine, spermidine and spermine) are aliphatic amines that are produced by the action of ornithine decarboxylase (ODC) in a rate-limiting and protein kinase C (PKC)-regulated step. Because high levels of polyamines are found in the synovial fluid of arthritic patients, the aim of the present study was to identify the role of peripherally produced polyamines in a model of inflammatory pain induced by adjuvant. The subcutaneous injection of Complete Freund's adjuvant (CFA, 50 μL/paw) caused the development of mechanical allodynia and edema. Moreover, it increased ODC expression and activity and PKC activation. Administration of the selective ODC inhibitor DFMO (10 μmol/paw) attenuated the development of allodynia and edema and decreased ODC activity in both control and CFA-treated animals. Furthermore, administration of the PKC inhibitor GF109203X (1 nmol/paw) reduced allodynia and ODC activity in animals injected with CFA. A subcutaneous injection of putrescine (10 μmol/paw), spermidine (3–10 μmol/paw) or spermine (0.3–3 μmol/paw) into the rat paw also caused mechanical allodynia and edema. The present results suggest that endogenously synthesized polyamines are involved in the development of nociception and edema caused by an adjuvant. Moreover, polyamine production in inflammatory sites seems to be related to an increase in ODC activity stimulated by PKC activation. Thus, controlling polyamine synthesis and action could be a method of controlling inflammatory pain.

1. Introduction

Polyamines (putrescine, spermidine and spermine) are ubiquitous and small aliphatic amines present in almost all cells [1]. Putrescine is formed from the decarboxylation of ornithine by ornithine decarboxylase (ODC; E.C 4.1.1.17) in a rate-limiting step in polyamine synthesis [2,3]. Putrescine is converted into spermidine and spermine by spermidine synthase and spermine synthase, respectively, which are two aminopropyl transferases [1,2]. ODC is a protein with a short half-life (10 min-1 h in mammals), and its activity and expression are highly regulated by the availability of polyamines and protein kinase C (PKC) [4–6]. Besides being endogenously synthesized, an exogenous supply of polyamines is provided through dietary intake and intestinal absorption from bacterial metabolism [7].

Polyamines are involved in several biological processes, including the control of neuronal excitability and memory improvement and learning in the central nervous system [1,8]. Previous studies have shown that a diet deficient in polyamines and supplemented with an antibiotic (to reduce the level of microflora-derived polyamines) relieves the hyperalgesia induced by incisions, inflammation or neuropathy in rats. This effect seems to be mediated by N-methyl-D-aspartate receptor (NMDA) in the spinal cord [9,10]. Accordingly, some studies have shown that spermine administered in the spine (by the intrathecal route) produces nociceptive behavior in rodents [11,12]. Apart from their pro-nociceptive action at the spinal cord, the role of peripheral polyamines in pain is unknown.

Interestingly, increased levels of polyamines are found in tissues and synovial fluid from patients with osteo-, rheumatoid, posttraumatic and infectious arthritides [13,14]. Moreover, it has been demonstrated that the activity and expression of ODC are increased in colonic tissue from Inflammatory Bowel Disease (IBD) patients [15]. These findings indicate that endogenously produced polyamines could play a role in inflammatory pain. Therefore, the present study investigated the role of peripheral, endogenous polyamines in nociception and edema in the early phase of CFA-induced inflammation.

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2. Material and methods

2.1. Animals

Experiments were performed on adult male Wistar rats (weight 250–300 g) bred in our animal house. The animals were housed in a controlled temperature (22 ± 2 °C) with a 12 h light/dark cycle. They were given standard lab food and water *ad libitum*. The animals were habituated to the experimental room for at least 30 min before the experiments. The experiments were performed in accordance with current ethical guidelines for the investigation of experimental pain in conscious animals [16]. The number of animals and the intensities of the noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments. The Committee on the Use and Care of Laboratory Animals at our university approved this study (no. 23081.012331/2009-81).

2.2. Drugs and treatments

The following drugs and chemicals were used in this study: putrescine, spermidine, spermine, DL-α-difluoromethylornithine-hydrochloride hydrate (DFMO, 50 μl/paw, an ODC inhibitor), a PKC inhibitor (GF109203X, 50 μl/paw), phosphate-buffered saline (PBS), and Complete Freund's Adjuvant (CFA, 1 mg/ml of heat-killed *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide monooleate). All drugs and chemicals were obtained from Sigma (St. Louis, USA). The drug solutions were prepared in PBS except for GF109203X, which was prepared in 0.1% of dimethyl sulphoxide (DMSO).

To assess the role of peripheral, endogenous polyamines or PKC activation in inflammatory pain, CFA (50 μl) was administered subcutaneously (s.c.) under the plantar surface of the right hind paw, and a separate group of animals received an s.c. injection of vehicle (PBS). Different groups were also pre-injected s.c. with the selective ODC inhibitor DFMO (1–10 μmol/paw) or the PKC inhibitor GF109203X (1 nmol/paw) 1 h before CFA administration. Higher doses of these drugs were not used because of their solubility limits.

To determine whether exogenously administered polyamines were capable of producing nociception or edema, putrescine, spermine or spermidine (50 μl, 0.003–10 μmol/paw) was also administered under the plantar surface of the right hind paw s.c., and a separate group received an s.c injection of vehicle (PBS). Higher doses of polyamines were not used because they elicited spontaneous nociception (observed as hind paw licking, lifting and shaking/flinching), which made nociception measurements difficult.

The effects of the drugs on nociception and edema were assessed from 0.5 to 24 h after drug administration. The drug doses and times of administration were based on pilot studies and literature [17].

2.3. Nociception assessment

Mechanical allodynia was measured as described previously by Chaplan et al. [18] and was considered an indicator of nociception. Rats were placed individually in clear Plexiglas boxes (9 cm × 7 cm × 11 cm) on elevated, wire-mesh platforms to access the ventral surface of the hind paws. The paws were touched with one in a series of seven von Frey hairs (6–100 g). The von Frey hairs were applied perpendicular to the plantar surface of the paws with sufficient force to cause a slight buckling against the paws and were held for approximately 2 s. The 50% withdrawal threshold was determined using the up-and-down method of Dixon [19]. In this paradigm, testing was initiated with the 15-g hair. Stimuli were always presented consecutively, whether ascending or descending. Withdrawal thresholds were verified at several time points after polyamine or CFA injection (from 0.5 to 24 h) and were compared with baseline values (before drug administration).

2.4. Edema formation assessment

The edema induced by different agents was considered as the increase in paw thickness, measured by a digital caliper (Mytutoio, Japan), as described previously by Milano et al. [20]. Paw thicknesses were verified at several time points after polyamine or CFA injection (0.5–24 h) and compared to baseline values (before drug administration).

2.5. ODC activity

After behavioral observation, paw-skin samples proximal to the point of injection were collected to perform ODC activity analysis according to Tabib [21] with minor modifications. The samples were homogenized in buffer (10 mM Tris–HCl, pH 7.5) containing 2.5 mM of DL-dithiothreitol (DTT) and 0.1 mM of ethylenediaminetetraacetic acid (EDTA). Following a 10-min incubation at 4 °C, the homogenates were centrifuged at 35,000 × *g* for 45 min at 4 °C, and the supernatants were collected. The protein in the supernatants was measured using the Bradford [22] method. To perform these reactions, 200 μl (0.5 mg/ml of protein) of the supernatants was added to assay buffer (1 M Tris–HCl (pH 7.5), 250 mM of DTT, 2 mM of pyridoxal phosphate, 20 mM of L-ornithine, and 0.1 m of Ci/ml L-[1-^{14}C]-ornithine), and the mixtures were incubated at 37 °C for 30 min in capped-glass tubes. A filter-paper humidifier was attached to the top of each tube in 0.25 ml of 1 M hyamine hydroxide. Sulphuric acid (250 μL, 5 M) was added to the reactions, and the mixtures were incubated for 30 min at 37 °C. The filter papers were then collected to measure the $^{14}CO_2$ released from [^{14}C]-ornithine. ODC activity was expressed as $^{14}CO_2$%, compared with the control group (PBS-treated paws). The mean activity of ODC in the control group was 7.1 ± 0.7 pmol/min/mg protein.

2.6. ODC expression

We also performed Western blot analysis to verify the expression of ODC protein in the paw-skin samples. The assay was performed as described previously [17] with minor modifications. The paw tissues were homogenized in 300 μl of ice-cold buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM NaF, 10 μg/ml aprotinin, 10 mM β-glycerolphosphate, 1 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM DTT and 2 mM of sodium orthovanadate). After centrifugation (13,000 × *g* for 30 min at 4 °C), the supernatants containing the cytosolic fraction were collected. The protein contents were determined by the method of Bradford [22] using bovine serum albumin (BSA) as the standard. Protein (70 μg) as mixed in loading buffer (200 mM Tris, 10% glycerol, 2% SDS, 2.75 mM β-mercaptoethanol and 0.04% bromophenol blue) and boiled for 5 min. Proteins were separated in 12% sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gels and transferred to polyvinylidene difluoride (PVDF) membranes according to the manufacturer's instructions (PerkinElmer, USA). Proteins were stained on the PVDF membrane with a solution of 0.5% actin and 1% glacial acetic acid in water, and this served as the loading control [23]. The membranes were then dried, scanned and quantified with the PC version of Scion Image. The membranes were washed, blocked with 1% BSA in TBS-T (0.05% Tween 20 in Tris-borate saline) and incubated for 10 min with diluted (1:150), primary antibodies against ODC (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were processed using a SNAP i.d. system (Millipore, USA). Blots